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ORIGINAL ARTICLE BACTERIOLOGY

Extension of the *Legionella pneumophila* sequence-based typing scheme to include strains carrying a variant of the *N*-acylneuraminate cytidylyltransferase gene

M. Mentasti[1], A. Underwood[2], C. Lück[3], N. A. Kozak-Muiznieks[4], T. G. Harrison[1] and N. K. Fry[1]

1) *Respiratory and Vaccine Preventable Bacteria Reference Unit,* 2) *Applied Laboratory and Bioinformatics Unit, Public Health England, London, UK,* 3) *Institut für Medizinische Mikrobiologie und Hygiene, TU Dresden, Dresden, Germany and* 4) *Centers for Disease Control and Prevention, Atlanta, GA, USA*

Abstract

Sequence-based typing (SBT) combined with monoclonal antibody subgrouping of *Legionella pneumophila* isolates is at present considered to be the reference standard during epidemiological investigation of Legionnaires' disease outbreaks. In some isolates of *L. pneumophila*, the seventh allele of the standard SBT scheme, *neuA*, is not amplified, because a homologue that is refractory to amplification with the standard *neuA* primers is present. Consequently, a complete seven-allele profile, and hence a sequence type, cannot be obtained. Subsequently, primers were designed to amplify both *neuA* and the homologue, but these yielded suboptimal sequencing results. In this study, novel primers specific for the *neuA* homologue were designed and internationally validated by members of the ESCMID Study Group for *Legionella* Infections at national and regional *Legionella* reference laboratories with a modified version of the online *L. pneumophila* sequence quality tool. To date, the addition of the *neuAh* target to the SBT protocol has allowed full typing data to be obtained for 108 isolates of 11 different serogroups, namely 1, 2, 3, 4, 5, 6, 7, 8, 10, 13, and 14, which could not previously be typed with the standard SBT *neuA* primers. Further studies are necessary to determine why it is still not possible to obtain either a *neuA* or a *neuAh* allele from three serogroup 11 isolates.

Keywords: ESGLI, *Legionella pneumophila*, *neuA*, *neuAh*, sequence-based typing
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Corresponding author: M. Mentasti, Respiratory and Vaccine Preventable Bacteria Reference Unit, Public Health England, 61 Colindale Avenue, London NW9 5EQ, UK
E-mail: massimo.mentasti@phe.gov.uk

Introduction

Legionellae are natural pathogens of protozoa that can also infect humans, causing a potentially lethal form of pneumonia known as Legionnaires' disease (LD). The infection is typically acquired by inhalation of aerosols from colonized water systems of both natural and artificial origin. In 2011, 4897 LD cases were reported by EU member states, Iceland, and Norway; although many different *Legionella* species can be isolated from environmental sources, among 600 culture-confirmed LD cases, *Legionella pneumophila* alone caused 96% of LD infections and serogroup 1 strains were isolated from 85% of these [1].

During LD outbreak investigations, clinical and environmental *L. pneumophila* isolates must be typed to identify the source of the infection. Sequence-based typing (SBT) is the internationally accepted typing method developed by members of the ESCMID Study Group for *Legionella* Infections (ESGLI, formerly the European Working Group for *Legionella* Infections). SBT consists of the DNA sequence determination of defined regions of seven genes (namely *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) in order to obtain an allelic profile and a sequence type (ST) number, which are then used together

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with serogrouping and monoclonal antibody subgrouping data to compare clinical strains with those isolated from different environmental sources [2,3]. In some isolates, it is not possible to amplify *neuA*, a gene coding for *N*-acylneuraminate cytidyltransferase, one of the enzymes involved in lipopolysaccharide (LPS) biosynthesis [4]; consequently a complete allelic profile and an ST number cannot be obtained. Farhat *et al.* [5] sequenced the LPS locus from Dallas-1E, the serogroup 5 type strain of *L. pneumophila*, and showed that a variant with 68% homology with *neuA*, and thus not amplified by the standard SBT primers, is present. Degenerate primers able to amplify and sequence both *neuA* and the *neuA* homolog (*neuAh*) were described. However, when they were brought into routine use, it was found that modification of PCR conditions was required for some strains, and even then suboptimal amplification and sequencing results were observed. Moreover, degenerate primers are not recommended for direct typing on DNA samples extracted from clinical specimens, a technique that is becoming increasingly important during LD outbreak investigations [6,7].

In this study, novel primers specific for *neuAh* were designed and subsequently internationally validated by ESGLI members. These primers have been shown to yield PCR products from a wide range of different strains under the same PCR conditions, and to give improved sequencing results in comparison with those previously described [5]. Furthermore, they have been used effectively in direct typing from clinical samples.

Materials and Methods

Bacterial strains

A panel of 47 *L. pneumophila* strains isolated in Europe, North America, Africa, and Australia, belonging to serogroups 2, 3, 4, 5, 6, 7, 8, 10, 11, 13, and 14, including ten type strains from the National Collection of Type Cultures (Public Health England, UK), that failed to give a *neuA* product with the standard SBT primers were tested (Table 1). Strains belonging to the remaining serogroups, namely 1, 9, 12, and 15, were not included, because amplification of the *neuA* target with the standard SBT primers was always successful by the completion of this study. Five further strains, namely EULV 2665, EULV 3028, EULV 6671, EULV 6861, and EULV 6862 (Table 1), distributed as part of the ESGLI *L. pneumophila* SBT Proficiency Panels between 2010 and 2012, were tested worldwide by ESGLI members at national and regional *Legionella* reference laboratories, in order to complete the validation of the novel *neuAh* primers.

DNA extraction

Strains were inoculated onto buffered charcoal yeast extract agar (Oxoid, Basingstoke, UK) and incubated at 37°C for 48–72 h in a moist atmosphere; two or three single colonies per strain were pelleted, and genomic DNA was extracted with the InstaGene matrix (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Extracted samples were centrifuged at 15 000 ***g*** for 5 min, and the supernatant was used as a template for PCR reactions.

Bioinformatic analysis and primer design

Nucleotide sequences of *neuA* (including 100 bp upstream and downstream of the gene) of the Corby, Lens, Paris and Philadelphia *L. pneumophila* serogroup 1 strains were obtained from the LegioList database (http://genolist.pasteur.fr/LegioList/), and that of the *neuAh* found in Dallas-1E, the *L. pneumophila* serogroup 5 strain, was obtained from GenBank (Accession number: FN256429). Sequences were aligned by the use of ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/), and primers were designed with Primer3 (http://frodo.wi.mit.edu/).

PCR assays

Amplification was performed by PCR in a final volume of 20 μL with 10 pmol of each primer. Two microlitres of supernatant from InstaGene extracts was used as DNA template, and 2 μL of DNA extracted from *L. pneumophila* Dallas-1E (NCTC 11405) and 2 μL of sterile PCR-grade water (Sigma-Aldrich, St. Louis, MO, USA), respectively, were used as positive and negative controls in all PCR experiments. First, 1 U of *Taq* DNA polymerase (Life Technologies, Carlsbad, CA, USA) was used in a DNA Engine (MJ Research, St. Bruno, Canada) under the following conditions: 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 50°C, and elongation for 30 s at 72°C; an initial denaturation step (5 min at 95°C) and a final elongation step (10 min at 72°C) were also included. Amplification was then repeated with a Fast PCR Mix (Life Technologies) in a Veriti thermocycler (Life Technologies) under the following conditions: 35 cycles of denaturation for 10 s at 95°C, annealing for 10 s at 55°C, and elongation for 20 s at 70°C; an initial denaturation step (1 min at 95°C) and a final elongation step (1 min at 72°C) were also included.

Gel electrophoresis

Two microlitres from each PCR reaction was analysed by electrophoresis in 2% E-gels (Life Technologies), and the results were visualized under UV light with the InGenius gel documentation system (Syngene, Cambridge, UK).

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TABLE 1. List of *Legionella pneumophila* strains included in this study

Strain	NCTC	Country	Source	Serogroup	Allelic profile	ST	*neuAh* length (bp)
H080160264		England	E	2	2, 10, 4, 28, 4, 4, 207	1302	696
ZS-HK 1 (4)		Czech Republic	E	3	2, 10, 15, 28, 4, 3, 207	1390	696
Los Angeles-1	11233	USA	C	4	11, 14, 16, 25, 7, 13, 206	1334	696
H100200318		England	E	4	5, 1, 22, 30, 6, 10, 203	1324	696
H080500042		England	E	4	5, 1, 22, 30, 6, 10, 203	1324	696
Dallas-1E	11405	USA	E	5	11, 14, 16, 18, 15, 13, 201	1300	699
H073240536		SHIP	C	5	11, 14, 16, 31, 15, 13, 210	1327	696
H073340034		SHIP	C	5	11, 14, 16, 31, 15, 13, 210	1327	696
H073280012		SHIP	E	5	11, 14, 16, 31, 15, 13, 210	1327	696
H073420023		SHIP	E	5	20, 26, 27, 34, 46, 27, 212	1328	696
H073340593		SHIP	C	5	11, 14, 16, 31, 15, 13, 210	1327	696
H042740084		England	E	6	6, 10, 22, 3, 21, 3, 207	1322	696
H093620216		England	E	6	2, 10, 3, 3, 9, 14, 207	1337	696
Chicago-8	11984	USA	E	7	2, 6, 17, 14, 12, 8, 211	1319	696
Concord-3	11985	USA	C	8	8, 1, 22, 30, 6, 10, 203	1320	696
03L113-2		Australia	E	8	13, 11, 4, 9, 23, 16, 214	1321	699
H064160534-4		England	E	8	5, 1, 22, 30, 6, 10, 203	1324	696
LC0569		England	C	8	3, 10, 1, 28, 14, 9, 207	1326	696
LC0606		England	E	8	3, 10, 1, 28, 14, 9, 207	1326	696
LC6546		England	C	8	12, 8, 11, 20, 40, 12, 216	1332	696
LC0467		England	C	8/10	3, 10, 1, 28, 14, 9, 207	1326	696
LC0831		England	C	8/10	5, 2, 22, 10, 6, 25, 203	1358	696
LC0850		England	C	8/10	3, 10, 1, 28, 14, 9, 207	1326	696
H062620006		England	C	10	6, 10, 3, 28, 9, 4, 207	1323	696
H064160536-3		England	E	10	7, 6, 3, 20, 13, 11, 205	1325	696
H070300486		Unknown	C	10	3, 10, 1, 28, 14, 9, 207	1326	696
H080200574		England	E	10	1, 4, 3, 5, 50, 1, 213	1329	699
H082960038		England	E	10	22, 4, 3, 8, 1, 15, 204	1391	696
H083580006		England	E	10	6, 35, 38, 42, 1, 14, 207	1336	696
H102700702		England	E	10	7, 6, 17, 28, 13, 11, 207	1288	696
LC0395		Belgium	E	10	2, 10, 3, 28, 9, 4, 207	1333	696
LC1132		England	C	10	2, 10, 3, 28, 9, 14, 207	1333	696
LC6813		England	E	10	2, 10, 3, 28, 9, 14, 207	1333	696
LC6817		England	E	10	2, 10, 3, 28, 9, 14, 207	1333	696
H112940222		England	C	10	2, 6, 17, 28, 13, 3, 207	1330	696
797-PA-H	12179	USA	C	11	12, 17, 11, 10, 5, 12, F	0	NA
LC4339		England	C	11	2, 10, 14, 28, 2, 2, F	0	NA
MO-296251110		Italy	C	11	21, 27, 28, 23, 15, 29, F	0	NA
82A3105	12181	USA	C	13	6, 10, 5, 10, 9, 1, 209	1318	699
LC5695		England	C	13	6, 10, 14, 10, 33, 1, 209	1331	699
1586-SCT-H	12174	Scotland	C	14	5, 1, 22, 30, 6, 10, 203	1324	696
1353799		South Africa	E	2–14	16, 21, 12, 19, 31, 21, 215	1317	699
1353802		South Africa	E	2–14	16, 21, 12, 19, 31, 21, 215	1317	699
1353819		South Africa	E	2–14	16, 21, 12, 19, 31, 21, 215	1317	699
MICU-B	12271	USA	E	ND	14, 18, 8, 18, 28, 19, 201	1335	699
U7W	12272	USA	E	ND	14, 18, 8, 18, 28, 19, 201	1335	699
U8W	12273	USA	E	ND	14, 18, 8, 18, 28, 19, 201	1335	699
EULV 2665		England	C	10	3, 10, 1, 28, 14, 9, 207	1326	696
EULV 3028		SHIP	C	5	11, 14, 16, 31, 15, 13, 210	1327	696
EULV 6671		England	E	10	6, 35, 38, 42, 1, 14, 207	1336	696
EULV 6861		Greece	E	3	3, 13, 1, 3, 14, 9, 207	1341	696
EULV 6862		Greece	E	10	3, 13, 1, 28, 14, 9, 207	1392	696

C, clinical; E, environmental; F, *neuA* amplification failed with SBT primers; NA, not applicable; ND, not determined; SHIP, infection acquired when travelling on cruise ship; ST, sequence type; Unknown, more than one country visited during the incubation period; 8/10, serogroup 8/serogroup 10 cross-reactive; 2–14, non-serogroup 1.

Sequencing

A 10-μL aliquot of each positive PCR reaction was sent together with the relevant primers to the Genomic Services Unit at Public Health England (London, UK). Purification of PCR products was performed with a magnetic bead system (Beckman Coulter, Pasadena, CA, USA), and DNA was then sequenced with an ABI platform (3730XL DNA Analyzer; Life Technologies).

Sequence analysis

Trace files obtained from the Genomic Services Unit were analysed with SeqScanner (Life Technologies), BioNumerics 6.1 (Applied Maths, Sint-Martens-Latem, Belgium) and the *L. pneumophila* sequence quality tool (SQT) (http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi).

Results and Discussion

First set of primers

A first set of primers, neuA_L (5′-CAATTCCATCCAGAAAAAAG-3′) and neuA_R (5′-ACTCTTTGGCTGCATAAA-3′), was designed on the basis of the alignment of the five *L. pneumophila* type strains, as described earlier. These primers were used to amplify a fragment of 858–861 bp (in some strains, a triplet deletion was observed) containing *neuAh* (699 bp in Dallas-1E) from all of the strains listed in Table 1. A fragment of the expected size was obtained for 41 of 47 *L. pneumophila* strains tested. No amplification was obtained for H073420023 (serogroup 5), H080200574 (serogroup 10), H112940222 (serogroup 10), 797-PA-H (serogroup 11), LC4339 (serogroup 11), and MO-296251110 (serogroup 11).

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Second set of primers

A second set of primers, neuAh_L (5′-ATCCAGCAGTTTTTAMAAATTTAGG-3′) and neuAh_R (5′-TGGCTGCATAAAYTAATTCTTTAGCCA-3′), was designed internally to neuA_L and neuA_R by use of the consensus of the *neuAh* sequences obtained with the first set of primers. The new primers were used to amplify a fragment of 791–794 bp containing *neuAh* from all of the strains listed in Table 1. A fragment of the expected size was obtained for 44 of 47 strains. No amplification was obtained for 797-PA-H (serogroup 11), LC4339 (serogroup 11), and MO-296251110 (serogroup 11).

Sequence quality

The quality of the trace files obtained with the novel primers was compared with that obtained with the primers published by Farhat *et al.* by use of SeqScanner (Life Technologies). The analysis showed a QV20+ value, defined as the total number of bases in the entire trace that have basecaller quality value of ≥20, close to 800 when neuAh_L and neuAh_R were used, as compared with values of <400 and <250, respectively, when neuA_up and neuA_do2 were used (Fig. 1). Considering that 354 bp is the length of the fragment used to determine the allele type for the *neuA* target, a QV20+ value of <250 is below that necessary to obtain a good-quality double-stranded consensus sequence across the entire allelic region.

neuA homologue

As previously described by Farhat *et al.* [5] *neuAh* exists in at least two variants with different lengths. In 11 of the 44 strains (including Dallas-1E), the gene was 699 bp in length, and encoded a protein of 232 amino acids, as in the *neuA* amplified by the standard SBT primers. The GAA triplet present at positions 499–501 in Dallas-1E (corresponding to glutamic acid at position 167 in the encoded protein) was absent in the remaining 33 strains (Table 1). The start codon is also distinct: ATG in the standard *neuA*, and GTG in the 699-bp variant (as in Dallas-1E), and TTG in the 696-bp variant (as in Los Angeles-1). The fact that GTG (normally encoding valine) and TTG (normally encoding leucine) can be used by prokaryotes as start codons instead of ATG has been reported previously [8].

Serogroup 11

No *neuA* or *neuAh* PCR product was obtained for 797-PA-H, LC4339, and MO-296251110. As all of these three strains belong to serogroup 11, and they are the only serogroup 11 strains available to the authors, it was decided to investigate this issue further. New primers, neuA_L2 (5′-GATACTTTATACGATGGTCAGCCTATTAG-3′) and neuA_R2 (5′-GCATTATTTTTTTGGTATTCCGCTTTT-3′), were designed, this time externally to neuA_L and neuA_R, on the basis of the alignment between the regions upstream and downstream of Dallas-1E *neuAh* and the correspondent *neuA* regions found in the Corby, Lens, Paris and Philadelphia strains. Amplification was performed as described above in a DNA Engine (MJ Research), but with 50°C for annealing and a 90 s extension time for 40 cycles. A PCR product of the expected size (1017 bp) was amplified from Dallas-1E, one fragment with a size smaller than expected (*c.* 800 bp) yielding weak fluorescence following electrophoretic analysis was amplified from 797-PA-H and LC4339, and no PCR product was obtained for MO-296251110. As expected, the sequenced Dallas-1E fragment showed a homology of *c.* 73% with the same fragment in the genome of the Paris strain according to blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The fragment sequenced from the two serogroup 11 strains instead showed

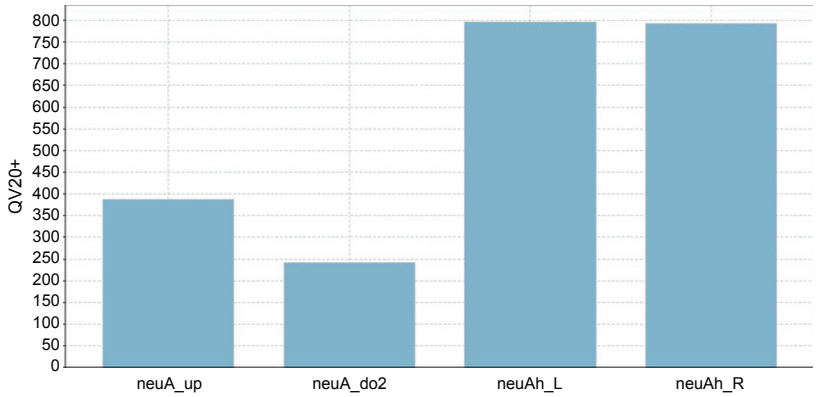

FIG. 1. Comparison of the QV20+ values of the *neuAh* trace files obtained from the *Legionella pneumophila* serogroup 5 Dallas-1E strain (NCTC 11405) with the Farhat *et al.* [5] primers (left) and the novel primers (right).

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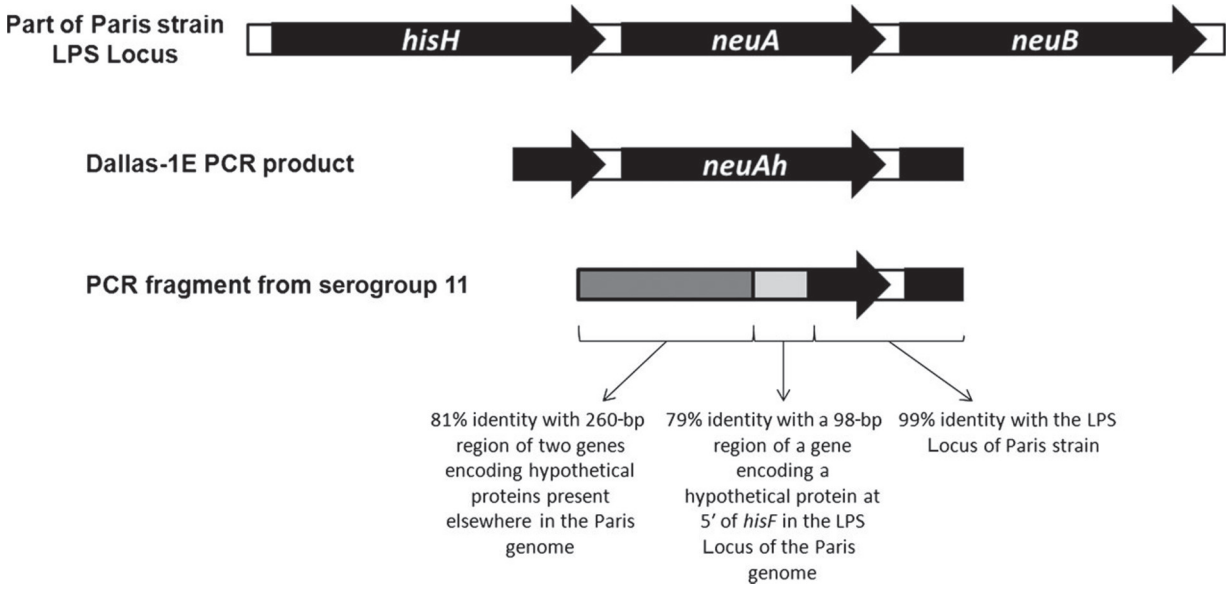

FIG. 2. Schematic representation of the blastn analysis of the amplicons obtained with the primers neuA_L2 and neuAh_R2, and comparison with part of the lipopolysaccharide (LPS) locus of the Paris strain of *Legionella pneumophila*.

FIG. 3. ClustalW analysis of the 50 *neuA* allele variants and the 22 *neuAh* allele variants so far described.

99% identity with the LPS locus of the Paris strain, but only at the 3′-terminus of the sequence (Fig. 2). The rest of the fragment showed identities of 79% and 81%, respectively, with two other genes of the Paris strain that are not part of the LPS locus. In order to determine whether this result was attributable to recombination or was a simple PCR artefact,

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the DNA sequences obtained from 797-PA-H and LC4339 were translated with the Translate Tool from the ExPASy website (http://web.expasy.org/translate). As a high number of stop codons were found, the recombination hypothesis appears to be more likely, because the presence of such codons suggests that these genes are not transcribed, and are thus free to accumulate mutations without these becoming lethal for the bacterial cell.

Modification of the *L. pneumophila* SQT

The SQT was modified to accept *neuAh* alleles and assign them to the *neuA* locus. Homologue alleles were designated starting from number 201, in order to differentiate them from *neuA* alleles. The creation of a new table to store the *neuAh* alleles in the *L. pneumophila* SBT database (www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php) was necessary, owing to the divergence of the *neuAh* and *neuA* sequences (Fig. 3). In addition, because, to date, three allelic length variants have been described (351, 354 and 357 nucleotides), the SQT was modified to allow for these differences from the other loci, which have a fixed length. To date, for the *neuA* locus, 22 *neuAh* alleles (*neuAh* 201 to *neuAh* 222) have been described and stored in the database, together with 50 *neuA* alleles (*neuA* 1 to *neuA* 50) (Fig. 3).

International validation

The novel primers neuAh_L and neuAh_R were tested worldwide by ESGLI members on five EULV strains that are refractory to the amplification of *neuA* (Table 1). Results were submitted by 35 laboratories for EULV 6861 and EULV 6862, by 19 laboratories for EULV 2665, and by 18 laboratories for EULV 6671 and EULV 3028 (Table 2). The expected *neuAh* allele was obtained by all testing laboratories for EULV 2665 (207), EULV 3028 (210), EULV 6671 (207), and EULV 6862 (207). EULV 6861 was correctly reported as *neuAh* 207 by 33 of 35 laboratories. Two laboratories erroneously obtained a *neuA* (12) allele for these strains. As this allele was consistent

TABLE 2. *neuAh* allele results and Phred scores obtained internationally by ESCMID Study Group for *Legionella* Infections (ESGLI) members on five EULV strains distributed as part of the ESGLI *Legionella pneumophila* SBT Proficiency Panel between 2010 and 2012

	EULV 3028		EULV 6671		EULV 2665		EULV 6862		EULV 6861	
Laboratory	*neuAh* allele	Phred score	*neuAh* allele	Phred score	*neuAh* allele	Phred score	*neuAh* allele	Phred score	*neuAh* allele	Phred score
1	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
2	NT	NT	NT	NT	NT	NT	207	89.49	207	86.04
3	210	90.00	207	90.00	207	90.00	207	90.00	207	90.00
5	NT	NT	NT	NT	NT	NT	207	90.00	207	90.00
6	210	90.00	207	90.00	207	90.00	207	90.00	207	90.00
8	NT	NT	NT	NT	NT	NT	207	90.00	207	90.00
9	NT	NT	NT	NT	NT	NT	207	90.00	207	82.01
11	NT	NT	NT	NT	NT	NT	207	90.00	207	90.00
12	210	89.44	207	90.00	207	90.00	207	90.00	207	90.00
14	210	90.00	207	90.00	207	90.00	207	89.92	207	90.00
15	NT	NT	NT	NT	NT	NT	207	90.00	207	89.83
16	210	89.96	207	90.00	NT	NT	207	90.00	207	90.00
20	210	89.87	207	90.00	207	89.99	207	89.94	207	89.99
23	210	89.25	207	90.00	207	87.38	207	90.00	207	90.00
24	210	89.84	207	88.57	207	88.82	207	88.30	207	88.24
25	210	90.00	207	90.00	207	90.00	207	90.00	207	90.00
26	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
27	NT	NT	NT	NT	NT	NT	207	90.00	12	NA
28	NT	NT	NT	NT	NT	NT	207	90.00	207	90.00
29	210	89.93	207	88.56	207	89.31	207	84.67	207	90.00
30	210	89.70	207	90.00	207	90.00	207	90.00	207	90.00
32	210	90.00	207	90.00	207	90.00	207	86.22	207	83.04
33	210	89.99	207	90.00	NT	NT	207	90.00	207	90.00
34	NT	NT	NT	NT	NT	NT	207	89.99	207	90.00
35	NT	NT	NT	NT	NT	NT	207	90.00	207	90.00
36	210	89.98	207	90.00	207	89.97	207	86.41	207	89.64
39	NT	NT	NT	NT	207	90.00	207	90.00	207	90.00
40	NT	NT	NT	NT	NT	NT	207	90.00	207	90.00
43	210	90.00	207	90.00	207	90.00	207	90.00	207	89.27
44	NT	NT	NT	NT	NT	NT	207	90.00	12	NA
46	NT	NT	NT	NT	NT	NT	207	90.00	207	90.00
47	210	90.00	207	90.00	207	90.00	207	88.23	207	90.00
48	NT	NT	NT	NT	NT	NT	207	80.20	207	80.76
49	210	90.00	207	90.00	207	90.00	207	90.00	207	90.00
51	NT	NT	NT	NT	207	90.00	207	89.98	207	89.92
52	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
53	NT	NT	NT	NT	207	90.00	207	90.00	207	90.00
54	NT	NT	NT	NT	207	90.00	207	90.00	207	90.00
55	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

NA, not applicable; NT, not tested.

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with another strain in the proficiency panel, this strongly suggests a contamination event, highlighting the fact that contamination issues can affect PCR-based typing schemes and cause the designation of new STs from allelic profiles that are not real. The two *neuA* (12) results were not taken into account in the final analysis. All participants obtained good-quality sequences, with Phred scores ranging from 80.20 to 90.00; in most cases, the score was equal to 90.00 (80 of 125 submitted *neuAh* alleles) or higher than 85.00 (115 of 125 submitted *neuAh* alleles).

Conclusions

In this article, we describe the modification of the internationally standardized *L. pneumophila* SBT protocol in order to include those strains of *L. pneumophila* for which the amplification of *neuA* is not successful because a homologue, *neuAh*, is present. Novel primers specific for *neuAh* were shown to provide superior results than those described previously by Farhat *et al.* Longer PCR fragments (791–794 bp vs. 431–434 bp) and better sequence quality (QV20+ *c.* 800 vs. <400) were obtained, greatly facilitating the designation of the *neuAh* alleles.

The presence of two variants of *neuAh*, one of 699 bp with GTG as the start codon and one of 696 bp with TTG as the start codon, was confirmed. In the case of all three available serogroup 11 strains, it was not possible to obtain either a full *neuA* or a full *neuAh* sequence. For two of these strains, i.e. 797-PA-H and LC4339, only a fragment with 99% homology in the 3′-region with the *neuA* found in the Paris strain was observed. The presence of several stop codons suggests a possible recombination event; however, sequencing of the LPS locus or of the whole genome should be performed in order to confirm this hypothesis. Farhat *et al.* did not include any serogroup 11 strain in their analysis, so this issue was not previously identified.

The *L. pneumophila* SQT was modified in order to recognize and process *neuAh* trace files generated with neuAh_L and neuAh_R, and to designate *neuAh* alleles starting from allele number 201 in order to differentiate them from the *neuA* alleles. The novel primers were tested worldwide by ESGLI members at national and regional *Legionella* reference laboratories with five *L. pneumophila* EULV strains. Following the satisfactory results obtained during this validation study, the novel *neuAh* primers have been officially incorporated into the SBT scheme of *L. pneumophila*, and are used in combination with the modified SQT to obtain a full seven-allele SBT profile, and hence an ST, when the amplification of *neuA* is not successful with the standard SBT primers. To date, *neuAh* data for 108 isolates of 11 different serogroups, namely 1, 2, 3, 4, 5, 6, 7, 8, 10, 13, and 14, comprising 22 distinct alleles, have been submitted to the international *L. pneumophila* database. Further studies are necessary to investigate why neither *neuA* nor *neuAh* are amplified from serogroup 11 strains.

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Transparency Declaration

The authors declare no conflicts of interest.

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